

Original Article

Fascin-1 is released from proximal tubular cells in response to calcineurin inhibitors (CNIs) and correlates with isometric vacuolization in kidney transplanted patients

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Abstract: Immunosuppression based on calcineurin inhibitors (CNIs) has greatly improved organ transplantation, although subsequent nephrotoxicity significantly hinders treatment success. There are no currently available specific soluble biomarkers for CNI-induced nephrotoxicity and diagnosis relies on renal biopsy, which is costly, invasive and may cause complications. Accordingly, identification of non-invasive biomarkers distinguishing CNI-induced kidney tubular damage from that of other etiologies would greatly improve diagnosis and enable more precise dosage adjustment. For this purpose, HK-2 cells, widely used to model human proximal tubule, were treated with CNIs cyclosporine-A and FK506, or staurosporine as a calcineurin-independent toxic compound, and secretomes of each treatment were analyzed by proteomic means. Among the differentially secreted proteins identified, only fascin-1 was specifically released by both CNIs but not by staurosporine. To validate fascin-1 as a biomarker of CNI-induced tubular toxicity, fascin-1 levels were analyzed in serum and urine from kidney-transplanted patients under CNIs treatment presenting or not isometric vacuolization (IV), which nowadays represents the main histological hallmark of CNI-induced tubular damage. Patients with chronic kidney disease (CKD) and healthy volunteers were used as controls. Our results show that urinary fascin-1 was only significantly elevated in the subset of CNI-treated patients presenting IV. Moreover, fascin-1 anticipated the rise of sCr levels in serially collected urine samples from CNI-treated pulmonary-transplanted patients, where a decline in kidney function and serum creatinine (sCr) elevation was mainly attributed to CNIs treatment. In conclusion, our results point towards fascin-1 as a putative soluble biomarker of CNI-induced damage in the kidney tubular compartment.

Keywords: Fascin-1, calcineurin inhibitors (CNIs), nephrotoxicity, biomarkers, transplant

Introduction

The calcineurin inhibitors (CNIs), cyclosporine A (CsA) and FK506, have been widely used in the prevention of allograft rejection since its introduction in the late 1970s and early 1980s, respectively. Even now, most of solid organ transplanted patients are under a CNI-based immunosuppressant pattern. Despite its benefits, CNIs produce several toxic side effects, the most relevant of which is renal toxicity. CNI-induced nephrotoxicity has been etiologically attributed to its vasoconstrictor action and direct cytotoxic effects on tubular epithelial

cells. Up to 76% of kidney transplanted patients show signs of CNIs nephrotoxicity after one year of transplantation, and up to 97% after ten years [1]. Histological findings associated to CNIs nephrotoxicity include arteriolopathy, tubular isometric vacuolization and thrombotic microangiopathy (TMA) in acute nephrotoxicity and arteriolar hyalinosis with interstitial fibrosis, tubular atrophy (IFTA), glomerulosclerosis and tubular microcalcifications in chronic nephrotoxicity. Nonetheless, CNIs side effects might be overestimated since most of these features are not exclusive of this pathology, making difficult to distinguish CNIs nephrotoxicity from

other injuries produced by factors like hypertension or ischemia-reperfusion [2].

Over the recent decades, many efforts have been done to identify biomarkers that could detect early loss of renal function. Parameters such as serum creatinine and proteinuria detect late kidney dysfunction and are highly unspecific. Proteins such as cystatin C [3], KIM-1 (kidney injury molecule-1) [4], FABP (fatty acid binding protein) [5, 6], NGAL (neutrophil gelatinase associated lipocalin) [7-9], among others [10-12], are being validated as novel biomarkers of kidney damage. Although these biomarkers could potentially anticipate an increase in creatinine levels and loss of kidney function, do not inform on whether the histological lesions are actually CNI-specific or not. At present, allograft biopsy and more specifically isometric vacuolization remains the best diagnostic option to detect kidney damage associated to CNIs treatment.

Considering all the above, there is an unmet need to find non-invasive soluble biomarkers for early detection of CNI-induced renal toxicity. To identify differentially secreted proteins that could indicate tubule specific injury due to CNIs, we have analyzed the conditioned media of CNI-treated cultured proximal tubular cells by a proteomic approach. To confirm their suitability as biomarkers, candidate proteins have been further validated by ELISA assays in blood and urine samples from kidney and pulmonary transplanted patients treated with CNIs.

Material and methods

Cell culture and treatment

HK-2 cells (ATCC n° CRL-2190; Manassas, VA, USA) were cultured as previously described [13]. Cells were seeded at 0.15×10^6 cells/mL and after 48 h, confluent cells were treated for 24 h with the indicated dose of CsA (Calbiochem, San Diego, CA, USA), FK506 (kindly provided by Astellas Pharma, Tokyo, Japan), staurosporine (Sigma Aldrich, Saint Louis, MO, USA) or vehicle alone in FBS-free medium.

Cytotoxicity assay

CsA, FK506 and staurosporine-induced cytotoxicity was measured by the LDH release assay using a commercial kit (Cytotoxicity Detection kit^{PLUS} (LDH), Roche, Mannheim, Germany) following instructions of the manu-

facturer. Each compound and dose was tested per triplicate in three different experiments performed on different days. Cytotoxicity (Percentage of LDH release) was calculated according to the following:

$$\% LDH_{release} = (LDH_{media} / LDH_{media} + LDH_{Lysate}) \times 100$$

The subtoxic dose for each condition was determined as the highest dose that did not induce cell death, in order to avoid the unspecific release of intracellular proteins.

2D electrophoresis

Cells seeded on 60.1 cm² culture plates (TPP, Trasadingen, Switzerland) were treated with subtoxic dose of each compound for 24 h in FBS-free medium. Ten ml of cell-free conditioned media were concentrated 200-fold to a final volume of 50 µl using 3 kDa cut-off Amicon centrifugal filters (Millipore, Tullagreen, Ireland). Sixty µg of each concentrated medium was mixed with rehydration solution (8 M urea, 2% CHAPS, 0.28% DTT, 0.5% IPG ampholine buffer pH 3-11 and 0.002% bromophenol blue) to a final volume of 125 µl and separated by isoelectric focusing in a 7-cm polyacrylamide gel with immobilized 3-11 pH gradient (IPGphor system, Amersham Biosciences, Freiburg, Germany). Gel strips with the focused proteins were equilibrated for 15 min in reducing SDS buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS and 1% DTT), followed by 15 min incubation in alkylating SDS buffer (SDS buffer with 4% iodoacetamide instead of DTT). The strips were placed on a 1 mm, 5 × 7 cm SDS-PAGE gel, and the second dimension was run in Tris-Tricine-SDS Buffer [14] in a miniprotean III electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA).

2-D differential analysis and mass spectrometry

Gels were stained with colloidal Coomassie blue [15], digitized with a calibrated densitometer (GS800; Bio-Rad Laboratories) and the resulting images analyzed using PDQuest 6.2 software (Bio-Rad Laboratories). Software spot assignment was checked and validated individually and spot intensity was normalized against the sum of intensity of the detected spots. The PDQuest software performed an automatic analysis using statistical criteria

(Student's *t*-test) to detect differentially secreted spots. Differential spots were excised, digested with trypsin and analyzed by MALDI-TOF on an ultraflex TOF-TOF (Bruker Daltonics, Bremen, Germany). Calibrated spectra were processed using FlexAnalysis 2.2 software (Bruker Daltonics). Spots that were not identified by MALDI-TOF were addressed by LC-ESI-MS/MS analysis on an esquire HCT IT mass spectrometer (Bruker Daltonics) coupled to a nano-HPLC (Ultimate; LC Packings, Amsterdam, The Netherlands). In this case, trypsin digested samples were loaded onto a 75-mm i.d., 15-cm PepMap nanoseparation column (LC Packings). MS/MS fragmentation (1.9 s, 100-2800 *m/z*) was performed on two of the most intense ions. The peak list was created with Data Analysis 3.4 software (Bruker Daltonics). Proteins were identified using Mascot v.2.2.04 (Matrix Science, London, UK) to search the international protein index (IPI)-Human 3.26 database, with a mass tolerance < 100 ppm for MALDI-TOF and 1.5 Da (precursor) and 0.5 Da (fragment) for MS/MS, allowing two missed cleavages for trypsin.

Immunoblotting

Five micrograms of cell extract and 35 µL of culture media were run on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Blots were blocked with 5% skimmed milk in PBST and probed with anti-fascin-1 antibody (HPA005723; Sigma-Aldrich) diluted 1:500 in blocking solution. Anti-MAPK antibody (06-182, Millipore) diluted 1:1000 in blocking solution was used as loading control.

Patients

Kidney transplanted patients, non-transplanted chronic kidney disease (CKD) patients and healthy individuals were included in the study (Supplementary Tables 1, 2 and 3). Inclusion criteria for kidney transplanted patients were: less than one year after transplantation and CNI-based immunosuppression combined with MMF and/or prednisone. Biopsy was indicated in patients with allograft dysfunction, proteinuria or as surveillance biopsy. None of these patients received intravenous immunoglobulins, iodine contrast or osmotic diuretics during the last month before biopsy. Banff criteria [16] were followed for histopathology damage assessment. All samples were collected shortly before biopsy to correlate the putative biomark-

ers with histology. Kidney transplanted patients were then further classified according to presence or absence of isometric vacuolization (IV). The evaluation and grading of IV was done following the schema described in [17]. In this study we considered positive IV when more than 5% of cortical tubules were involved. Inclusion criteria for non-transplanted patients were CKD in stage III and IV and a glomerular filtration rate between 15 and 60 ml/min (MDRD). Previous cardiovascular episodes were considered an exclusion criterion. Samples from pulmonary transplanted patients were used to validate the specificity of putative biomarkers. The inclusion criteria for this group were immunosuppressive regimen based on CNIs and normal kidney function before transplantation (Supplementary Table 4). Previous transplantation of any other organ was considered an exclusion criterion.

Sample collection and analysis

For urine samples, 30 mL of fresh urine were collected and centrifuged for 10 minutes at 1200 g (4°C) and supernatant conserved at -80°C. On the day of analysis, samples were thawed and centrifuged again for 10 minutes at 1200 g (4°C), and supernatants were concentrated to a final volume of 500 µl using 3 kDa cut-off Amicon centrifugal filters (Millipore). For blood samples, 5 ml of whole peripheral blood were collected and mixed with EDTA, and centrifuged for 10 minutes at 1200 g at room temperature. The resulting plasma fractions were aliquoted and stored at -80°C. Serum creatinine levels and proteinuria were analyzed by the Jaffe reaction [18] and the pyrogallol method, respectively. Blood levels of CNIs were evaluated by EMIT (Enzyme Multiplied Immunoassay Technique). All procedures followed the European Directives of Clinical Investigation and were approved by the Ethics Committee of Clinical Investigation of the Vall d'Hebron Hospital, Barcelona, Spain (PR(AG) 12/2010).

ELISA assays

Plasma and urine levels of fascin-1 and KIM-1 were determined with commercially available ELISA kits (fascin-1: E91757Hu, USCN Life Science Inc., Wuhan, PRC; KIM-1: DKM 100, R&D Systems, MN, USA) following the manufacturer instructions. Urinary levels of fascin-1 and KIM-1 were assessed diluting concentrated urine with MilliQ water 100 and 20 times,

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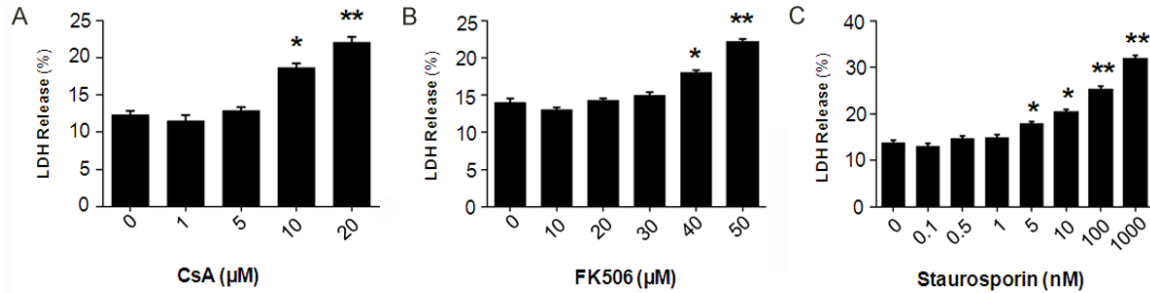


Figure 1. Cyclosporine A (CsA), FK-506 and staurosporine-induced cytotoxicity in HK-2 cells. Cultured HK-2 cells were treated for 24 h with increasing doses of CsA (A), FK-506 (B) and staurosporine (C) in FBS-free medium. Cytotoxicity was assessed by LDH assay. Each experiment was done three times with three replicates per condition. Results were analyzed by univariate ANOVA and differences between untreated cells and each experimental condition using Bonferroni contrasts (*, $P < 0.05$ and **, $P < 0.01$).

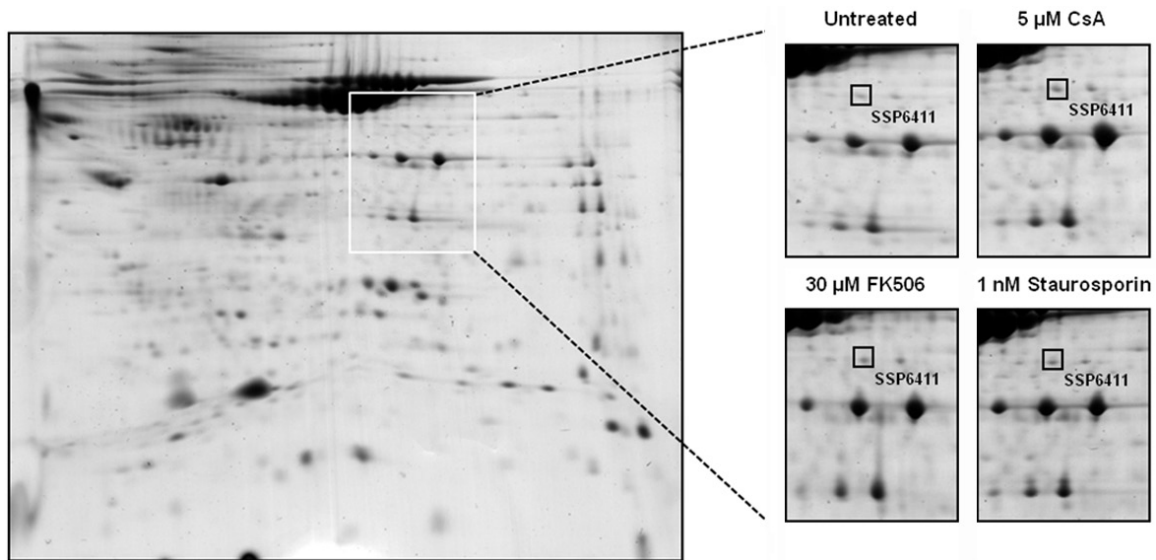


Figure 2. Fascin-1 levels are increased in the secretomes of HK-2 cells treated with CsA and FK-506 but not staurosporine. Cultured HK-2 cells were treated for 24 h with 5 μM CsA, 30 μM FK-506 and 1 nM staurosporine in FBS-free medium. Media were concentrated and 50 μg of protein were used to perform proteomic bidimensional analysis. Differential secreted proteins were identified by mass spectrometry. An enlarged view of the SSP6411 protein spot, corresponding to fascin-1 in each treatment condition, is shown.

respectively. ELISA assays were analyzed using a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Adjustment of the calibration curve was calculated by using a 4-parameter logistic regression (4PL). The results obtained in urine were normalized by the urinary creatinine levels.

Statistical analysis

Statistics was performed with GraphPad Prism 6 (GraphPad Software, Inc, CA, USA) and Statgraphics Plus 4.1 (Manugistics Inc., Herndon, VA, USA) software. The experiments performed in vitro were analyzed using Student's t-test. All tests used for ELISA data

analysis were non-parametric. For two data group comparison a Student's t-test with Welch correction was performed. When comparing more than two groups, a Kruskal-Wallis test was done followed by Dunn's multiple range comparison. Correlation analysis was performed using Pearson's test.

Results

Identification of proteins specifically secreted in response to subtoxic doses of CNIs

To identify early soluble biomarkers predicting kidney outcome under CNIs treatment, we aimed to identify proteins specifically secreted

Table 1. Mean intensity and fold change of the differentially represented spots identified in the secretomes of CsA, FK-506 and staurosporine-treated HK-2 cells

Spot ID		Spot intensities				Fold change		
		Ctrl	CsA	FK-506	Stauro	CsA vs Ctrl	FK506 vs Ctrl	Stauro vs Ctrl
3203	Ubiquitin carboxyl-terminal hydrolase isozyme L1	922.6 ± 29.1	1592.8 ± 13.9*	710.7 ± 32.6	1447.0 ± 49.1	1.7	0.8	1.6
6409	Plasminogen activator inhibitor-1	15042.0 ± 53.8	33362.5 ± 17.3*	15350.9 ± 28.1	14483.0 ± 51.3	2.2	1.0	1.0
7304	Glyceraldehyde-3-phosphate dehydrogenase	833.2 ± 63.7	2473.7 ± 17.9*	1385.2 ± 31.8	1474.3 ± 30.3	3.0	1.7	1.8
7303	Fructose-bisphosphate aldolase A	301.4 ± 27.7	678.5 ± 24.6*	690.9 ± 22.5*	959.0 ± 58.1	2.3	2.3	3.2
6411	Fascin-1	494.9 ± 43.3	1324.1 ± 115.1*	1109.5 ± 24.5*	510.1 ± 89.6	2.7	2.2	1.0
2206	Galectin-1	1702.8 ± 9.3	3133.7 ± 16.5	1723.9 ± 37.3	3139.5 ± 19.4*	1.8	1.0	1.8
3410	Fructose-bisphosphate aldolase A	1067.7 ± 78.8	610.4 ± 112.7	862.7 ± 84.7	3510.2 ± 8.0*	0.6	0.8	3.3
7408	Pyruvate kinase isozymes M1/M2	964.0 ± 104.9	1909.2 ± 77.9	449.8 ± 61.6	2683 ± 15.4*	2.0	0.5	2.8

Spot intensities of each condition were compared with control spot intensities using Student's t-test (*, $P < 0.05$).

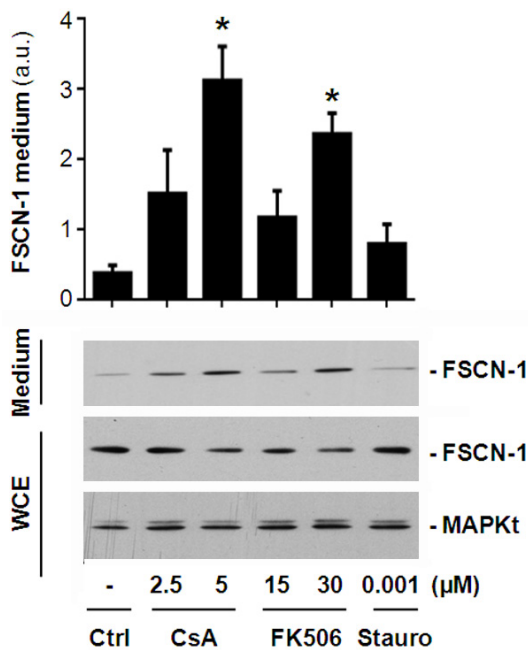


Figure 3. Western blot validation of fascin-1 levels in the conditioned media of CsA and FK-506 treated cells. Cultured HK-2 cells were treated during 24 h with 2.5 and 5 μ M CsA, 15 and 30 μ M FK-506 and 1 nM of staurosporine. Fascin-1 levels were detected by western blot using 35 μ l of medium in each condition and 5 μ g of whole cell extract (WCE). MAPKt was used as loading control of whole cell extracts. The experiment was performed three times with three replicates of each condition. Intensity of each band was assessed and data were analyzed by univariate ANOVA and Bonferroni's contrast (*, $P < 0.05$). Fascin-1 levels were significantly higher in CsA and FK-506 conditioned media.

by cultured tubule cells in response to CNIs. To distinguish regulated secretion from the unspecific leakage due to cell lysis caused by CNIs toxicity, we first analyzed the toxicity threshold of each drug. HK-2 cells were treated with increasing doses of the immunosuppressants CsA and FK506, and with staurosporine as positive control of cell death induction, and cytotoxicity was measured after 24 h by LDH assay. The subtoxic dose, defined as the highest dose not inducing significant LDH release to medium, was then determined for each compound. As shown in **Figure 1**, 5 μ M CsA, 30 μ M FK506 and 1 nM staurosporine were set up as the subtoxic doses upon 24 h treatment. HK-2 cells were then seeded on 60.1 cm² culture plates, treated as indicated, and 10 ml of each conditioned media were collected for proteomic analysis as stated in methods. A representative proteomic map of the secreted protein set, from now on secretome, is shown in **Figure 2**. Secretomes from each treatment condition were analyzed and compared with that from untreated control cells. Significantly altered protein spots were then collected and identified by mass spectrometry. Although several proteins were identified (see **Table 1**), only fascin-1, corresponding to spot 6411, was over represented (fold change > 1) in secretomes from both CsA and FK506-treated cells and was not altered (fold change \leq 1) by staurosporine. A zoom box depicting fascin-1 spot on each condition is shown in **Figure 2**. The pres-

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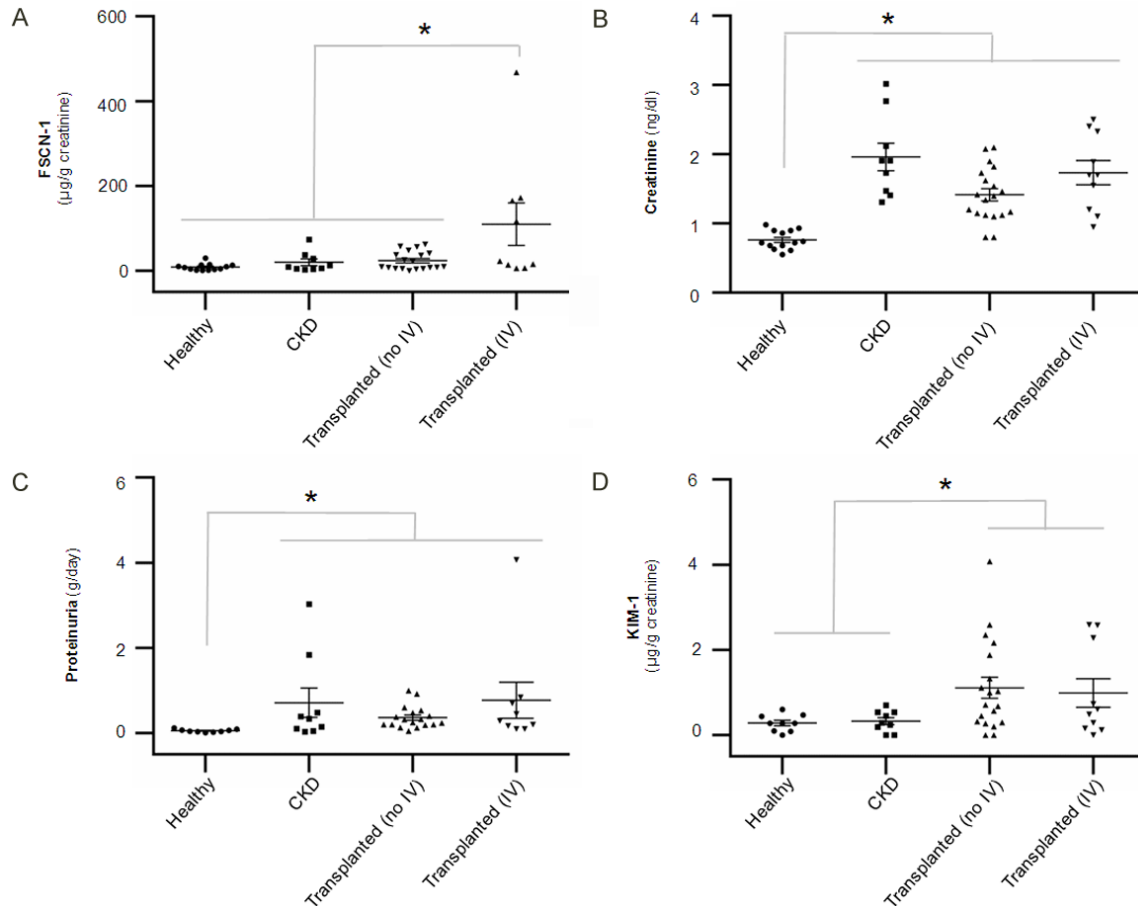


Figure 4. Urinary levels of fascin-1 are higher in kidney transplanted patients showing isometric vacuolization of tubular cells. Urinary fascin-1 (A), serum creatinine (B), proteinuria (C) and urinary KIM-1 (D) levels were analyzed in healthy volunteers, non-transplanted chronic kidney disease (CKD) patients and kidney transplanted patients presenting or not isometric vacuolization (IV) of tubular cells. Fascin-1 and KIM-1 levels were assessed using ELISA assays and the results were normalized with urinary creatinine levels. Each sample was tested twice (variation coefficient < 10%) and interplate controls were used. Data were analyzed using Kruskal-Wallis test and Dunn's contrasts (*, $P < 0.05$). Fascin-1 levels in urine were significantly higher in kidney transplanted patients showing isometric vacuolization of tubular cells (A).

ence of fascin-1 on the conditioned media of CsA and FK506 treated HK-2 cells was further validated by immunoblotting. As shown in **Figure 3**, levels of fascin-1 were correlating with the proteome data, since they were significantly higher in the conditioned media from CsA and FK506-treated cells compared to those levels in media from control and staurosporine-treated cells.

Analysis of fascin-1 levels in blood and urine from CNI-treated patients

Isometric vacuolization (IV) has been considered the main histological finding associated to CNI-induced toxicity upon the tubular compartment [19, 20]. To investigate the usefulness of

fascin-1 as a specific biomarker of CNI-induced tubular toxicity, we analyzed fascin-1 levels in serum and urine of kidney-transplanted patients under CNIs treatment presenting or not IV of tubule cells. As stated in the methods section, serum and urine samples from kidney transplanted patients were taken shortly before the kidney biopsy in order to accurately correlate fascin-1 levels with histological findings. Serum and urine from healthy volunteers were used as controls. To further corroborate that fascin-1 levels were specifically increased by CNIs treatment and not as a consequence of other kidney disease alterations, a third group of samples from non-transplanted CKD patients were included. Renal function markers includ-

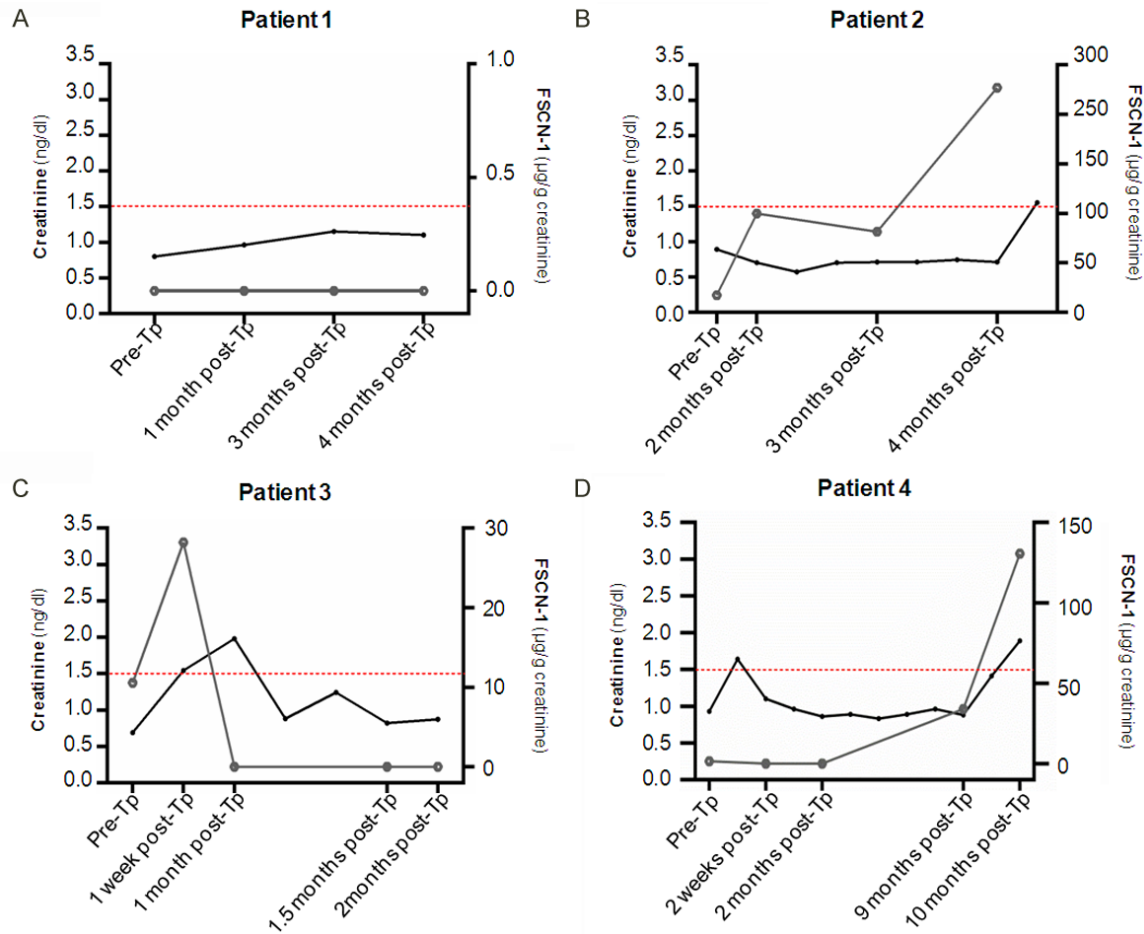


Figure 5. An increase in urinary fascin-1 levels precedes kidney dysfunction in lung transplanted patients. The graphs show the levels of fascin-1 in serial urine samples and the corresponding serum creatinine levels in lung transplanted patients 1 (A), 2 (B), 3 (C), 4 (D). Fascin-1 levels in urine were assessed using an ELISA assay and the results were normalized with urinary creatinine levels. Each sample was tested twice (variation coefficient < 10%) and interplate controls were used. Patients 2, 3 and 4 showed an increase of urinary fascin-1 levels preceding a loss of kidney function (B, C and D, respectively). Levels of creatinine serum above 1.5 mg/ml were considered altered kidney function. Tp: Transplantation.

ing creatinine and proteinuria, as well as the molecular marker of kidney injury KIM-1 were analyzed in those samples.

Our results indicate that urinary but not serum (results not shown) fascin-1 levels were statistically elevated in CNi-treated kidney-transplanted patients exhibiting IV, in comparison to healthy, CKD patients or CNi-treated kidney-transplanted patients without IV (**Figure 4A**). Moreover, both creatinine and proteinuria levels were significantly elevated in kidney-transplanted patients with or without IV, and also in CKD patients, indicating that the increase in fascin-1 levels found in kidney-transplanted patients with IV is not a mere consequence of altered renal function but a specific feature of

CNi's damage. Finally, KIM-1 levels were not statistically different between transplanted patients with or without IV, suggesting that kidney injury per se is not the responsible of fascin-1 upregulation.

Detection of fascin-1 in urine of lung transplanted patients

To investigate the potential role of urinary fascin-1 as an early biomarker of CNi-induced kidney damage, we analyzed fascin-1 levels in a time series of urinary samples from CNi-treated lung transplanted patients, where a decline in kidney function would be mainly attributed to CNi's treatment. Because kidney biopsy was not indicated in these patients, serum creati-

nine (sCr) levels were analyzed as suggestive of kidney endpoint toxicity of CNIs (see [Table S4](#)). Urinary fascin-1 levels were analyzed when urine samples were available. Our results show that lung transplanted patients with altered renal function (sCr > 1.5 mg/dl) exhibited a peak of urinary fascin-1 preceding the increase in sCr (**Figure 5B-D**). Moreover, patient 1, who presented normal renal function (sCr < 1.5 mg/dl), showed undetectable levels of urinary fascin-1 (**Figure 5A**). These results strengthen our previous data obtained in kidney transplanted patients and suggest that urinary fascin-1 levels could predict loss of kidney function due to CNI-induced nephrotoxicity.

Discussion

Cell lines of kidney origin represent a simplified scenario to explore mechanistic insights into renal physiopathology, assess new therapeutic strategies or discover potential biomarkers of kidney injury in a system not influenced by the higher order regulatory elements of the whole kidney. In this sense, secretome profiling of cultured cells has become a powerful strategy used to identify potential body fluid-accessible biomarkers. In the present work, we have used the human proximal tubule-derived cell line HK-2, which retain a phenotype indicative of well-differentiated PTCs [21], to identify potential protein biomarkers specifically released by proximal tubules in response to the cytotoxic action of CNIs in the tubule. By these means, we aim to discriminate the direct tubular toxicity of CNIs from the overlapping hemodynamic effects occurring in the kidney. Accordingly, we assume that proteins secreted into the extracellular media from cultured HK-2 cells exposed to CNIs are likely to resemble those secreted by human proximal tubular cells in the urine or blood of CNI-treated patients.

Both CsA and FK506 possess a very narrow therapeutic window and highly variable pharmacokinetics [22]. CNIs levels in renal tissue are much higher than in blood due to local accumulation [23, 24] and the final dose at which epithelial cells of the proximal tubule are exposed is difficult to determine. Experimentally, too low concentrations will have none or little effect on cells, whereas too high concentrations will trigger leakage of intracellular proteins into the medium through the damaged plasmatic membrane upon cell death. Taking

this into consideration, we set up the working dose of each drug as its highest subtoxic dose, as assayed by LDH release. As described [25-27], our results show that FK506 cytotoxicity is much lower than that of CsA, since higher doses of FK506 are required to achieve significant cell toxicity. To analyze the secretome profile of HK-2 cells under these conditions, proteomic maps of conditioned media from each treatment were compared and differentially secreted proteins were identified by mass spectrometry. It is noteworthy that the number of differentially secreted proteins identified in each condition is relatively low, likely related to the subtoxic drug doses used in this study. Among the proteins identified, we focused on those that were upregulated in both CsA and FK506 conditions since both drugs induce similar histological and functional injuries [28]. Moreover, candidate proteins whose levels were also affected by the apoptosis-inducer staurosporine were not considered by its potential involvement in non-specific cytotoxicity. Accordingly, only fascin-1 fulfilled these filtering criteria and was considered for further analysis.

In order to validate the suitability of fascin-1 as a biomarker of CNI-induced nephrotoxicity, fascin-1 levels were analyzed in serum and urine of CNI-treated kidney-transplanted patients. Assessment of CNI-induced nephrotoxicity has been challenging since the original description of the pathology and basically relies on the histological findings of renal biopsy. Systemic blood level monitoring of CNIs has proved to be of little help in predicting CNIs nephrotoxicity and specific and readily measurable soluble biomarkers are not currently available [29]. This mainly occurs because the interindividual susceptibility to CNIs nephrotoxicity is not directly related to the interindividual variability in systemic exposure to CNIs [19]. There are accumulating evidences that local renal concentrations of CNIs correlate with nephrotoxicity, but it is also true that the local levels in the kidney allograft are not directly related to the systemic levels. Accordingly, we have observed that neither the presence of isometric vacuolization nor fascin-1 levels correlated with systemic blood levels of CNIs ([Supplementary Figure 1A](#) and [1B](#), respectively). Considering all the above, fascin-1 levels were correlated with isometric vacuolization (IV), which represents the most specific histological finding of renal

biopsy indicative of CNIs damage upon the tubular compartment [19, 20]. Our results show that a significant increase in fascin-1 levels was only observed in the subset of CNI-treated patients who evidenced CNI-induced toxicity. Moreover, CKD patients presented clinical signs of kidney dysfunction with unaltered levels of fascin-1, indicating that fascin-1 is not merely released as a consequence of kidney injury, thus supporting the specificity of fascin-1 as a biomarker of CNI-induced nephrotoxicity.

In addition to its specificity, potential biomarkers must be able to anticipate kidney dysfunction. The temporal relationship between fascin-1 and the deterioration of renal function induced by CNIs was assessed in serially collected urinary samples of CNI-treated lung transplanted patients. Although no kidney biopsy was available from these patients for histological diagnosis, it is not unreasonable to assume that loss of kidney function and the corresponding increase in serum creatinine (sCr) levels would be almost exclusively attributable to CNIs treatment. Our results show that in CNI-treated patients with sCr levels over 1.5 mg/dl, a peak of fascin-1 levels was preceding the raising of sCr. Taken together, and despite the limited sample size of our patient cohorts, these results point towards urinary fascin-1 as a potential early biomarker of the direct and specific toxic effects of CNIs in kidney proximal tubule cells.

Fascins are actin-binding proteins that cross-link filamentous actin into tightly packed parallel bundles. These bundles are important for the organization and morphology of a wide range of sub-cellular structures that include dynamic and stable cell-surface protrusions that play diverse roles in cell-cell interaction and cell migration [30]. In this sense, fascins play a central role in the actin cytoskeleton reorganization that accompanies epithelial-to-mesenchymal transition (EMT) [31, 32], a process shown to occur in proximal tubule epithelial cells upon CNIs treatment [33]. This fact, together with our present results showing fascin-1 translocation from cytosol to the extracellular media, points towards a potential effect of CNIs modulating fascin-1 function and location. Since fascin-1 lacks an ER signal sequence targeting to the secretory pathway, its presence in the extracellular media must be explained by non-classical secretion mechanisms such as

secretory exosomes [34]. Accordingly, the presence of fascin-1 has been widely described in exosomes from many cancer cells [35-37]. As renal cells facing the urinary lumen, including tubular cells, actively release these vesicles, urinary extracellular vesicles have recently emerged as a potential source of soluble biomarkers [38]. Thus, it is conceivable that fascin-1 found in the extracellular media of CNI-treated cells or the urine of CNI-treated patients could result from exosomes released by tubular cells.

In conclusion, our data suggest that fascin-1 may represent a non-invasive urinary biomarker to assess tubular damage induced by CNIs. Early and specific detection of CNI-induced nephrotoxicity will allow in turn, adjustment of CNIs doses in order to achieve its optimal therapeutic effect without developing nephrotoxicity. To further assess the value of fascin-1 as a clinical biomarker, further clinical assays shall be validated in more extensive cohorts.

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Disclosure of conflict of interest

None.

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Fascin-1 is a potential biomarker of CNi-induced nephrotoxicity

Table S1. Individual clinical data of kidney transplanted patients' group. Data of this group are split attending to absence or presence of isometric vacuolization. Presence of isometric vacuolization was considered when more than 5% of tubular sections were involved. The medical indication of the allograft biopsy was acute allograft dysfunction, delayed graft function, proteinuria > 1 g/day or surveillance after 3 months of transplantation (MDRD-4 > 30 and proteinuria < 0.8 g/day)

KIDNEY TRANSPLANTED PATIENTS									
No Isometric Vacuolization									
Patient	Age (years)	time post-TP (months)	Creatinine (mg/dl)	Proteinuria (g/day)	FK-506 (ng/ml)	CsA (ng/mL)	Gender	Biopsy Indication	Banff
2	68.11	1.08	2.1	0.92	10.7		Male	Acute Graft Dysfunction	Normal
3	45.38	1.64	1.73	0.475	8.1		Male	Acute Graft Dysfunction	Normal
4	28.19	2.85	1.9	0.6	9.8		Male	Delayed Graft function	ABMR
1	67.77	3.64	1.17	1	5.2		Male	Surveillance	IFTA II
5	55.21	2.33	1.54	0.25	12.3		Male	Surveillance	IFTA I
6	32.22	2.98	0.8	0.4	8.6		Male	Surveillance	Borderline
7	71.28	3.08	1.82	0.38	11.8		Male	Surveillance	IFTA I
8	44.56	3.15	1.12	0.2	10.2		Female	Surveillance	Normal
9	61.46	3.18	1.1		7.6		Female	Surveillance	IFTA I
10	72.07	3.25	1.4	0.126	9.6		Male	Surveillance	IFTA I
11	36.73	3.54	1.46	0.048	11.6		Male	Surveillance	Normal
12	62.48	3.57	1.12	0.2	7.1		Male	Surveillance	IFTA I
13	69.92	3.7	1.42	0.318	11.6		Male	Surveillance	IFTA I
14	44.73	3.84	1.34	0.13	7.5		Male	Surveillance	Normal
15	70.34	3.87	1.62	0.327	9.3		Male	Surveillance	IFTA II
16	39.07	4.13	1.15	0.204		158	Female	Surveillance	IFTA I
17	39.07	4.36	0.8	0.53	11		Female	Surveillance	ABMR
18	48.02	4.49	1.2	0.241	10.9		Male	Surveillance	Normal
Mean	53.15	3.26	1.38 [‡]	0.37 [‡]	9.58	158.00			
SEM	3.53	0.21	0.09	0.06	0.48	--			
Isometric Vacuolization									
Patient	Age (years)	time post-TP (months)	Creatinine (mg/dl)	Proteinuria (g/day)	FK-506 (ng/ml)	CsA (ng/mL)	Gender	Biopsy Indication	Banff
1	64.9	3.8	2.5		10.8		Male	Acute Graft Dysfunction	ABMR
2	35.95	7.61	1.89	0.84	14		Male	Acute Graft Dysfunction	IFTA II
3	71.26	10.92	2.33	0.2	5.8		Male	Acute Graft Dysfunction	IFTA II
4	79.94	2.82	2.4	0.45	6.9		Male	Acute Graft Dysfunction	IFTA II
5	72.11	1.8	1.7	0.7		240	Male	Acute Graft Dysfunction	ATN
6	45.52	0.95	0.95	4.07	10.6		Female	Proteinuria	FSGS Recurrence
7	75.91	2.85	1.7	0.17	10.9		Male	Surveillance	Borderline
8	66.94	4.59	1.1	0.097	14.2		Female	Surveillance	Borderline
9	74.7	3.77	1.2	0.1	7.1		Female	Surveillance	Borderline
10	58.11	4.69	1.55	0.287	15.3		Male	Surveillance	IFTA II
Mean	64.53	4.38	1.73 [‡]	0.77 [‡]	10.62	240.00			
SEM	4.47	0.93	0.17	0.40	1.09	--			

ABMR: Antibody-mediated rejection, IFTA: interstitial fibrosis and tubular atrophy, ATN: acute tubular necrosis, Borderline: borderline rejection. FSGS: Focal segmental glomerulosclerosis. TP: Transplantation. [‡]Different from healthy volunteers group (Student's t-test with Welch correction, P < 0.05).

Fascin-1 is a potential biomarker of CNI-induced nephrotoxicity

Table S2. Individual clinical data of Chronic Kidney Disease (CKD) patients group

CHRONIC KIDNEY DISEASE (CKD) PATIENTS						
Patient	Age (years)	Creatinine (mg/dl)	eGFR (MDRD-4)	Proteinuria (g/day)	Gender	CKD Stage
1	63.7	2.12	31.82	0.338	Male	III
2	61.28	1.41	51.39	1.836	Male	III
3	36.39	1.91	44	0.102	Male	III
4	56.28	1.47	48.84	0.034	Male	III
5	41.31	2.77	25.56	3.031	Male	IV
6	56.19	1.73	30.56	0.145	Female	III
7	60.98	1.31	56.13	0.051	Male	III
8	34.83	3.02	17.78	0.39	Male	IV
9	65.19	1.91	26.45	0.483	Female	IV
Mean	52.91	1.96 ¹	36.95	0.71 ¹		
SEM	4.01	0.20	4.48	0.34		

¹Different from healthy volunteers group (Student's t-test with Welch correction, $P < 0.05$).

Table S3. Individual clinical data of healthy volunteers group

HEALTHY VOLUNTEERS				
Healthy volunteer	Age (years)	Creatinine (mg/dl)	Proteinuria (g/day)	Gender
1	34	0.98	Negligible	Male
2	29	0.86	Negligible	Female
3	29	0.9	Negligible	Female
4	26	0.93	Negligible	Male
5	34	0.9	Negligible	Male
6	24	0.72	Negligible	Female
7	32	0.63	Negligible	Female
8	31	0.68	Negligible	Female
9	32	0.68	Negligible	Female
10	51	0.74	Negligible	Male
11	40	0.55	Negligible	Female
12	50	0.72	Negligible	Male
13	59	0.61	Negligible	Female
Mean	36.23	0.76	--	
SEM	2.97	0.04	--	

Fascin-1 is a potential biomarker of CNi-induced nephrotoxicity

Table S4. Clinical data of lung transplanted patients group

LUNG TRANSPLANTED PATIENTS							
Patient 1				Patient 2			
Gender		Age (years)		Gender		Age (years)	
Male		45		Female		60	
Months from TP	Sample	Creatinine (mg/dl)	CsA (ng/ml)	Months from TP	Sample	Creatinine (mg/dl)	FK-506 (ng/ml)
Pre-TP	•	0.8	0	Pre-TP	•	0.89	0
1	•	0.96	206	2	•	0.7	15
3	•	1.15	415	3	•	0.71	8.1
4	•	1.1	276	4	•	0.71	14
				6		1.6	16.5
Patient 3				Patient 4			
Gender		Age (years)		Gender		Age (years)	
Male		33		Male		53	
Months from TP	Sample	Creatinine (mg/dl)	FK-506 (ng/ml)	Months from TP	Sample	Creatinine (mg/dl)	CsA (ng/ml)
Pre-TP	•	0.69	0	Pre-TP	•	0.93	0
0.25	•	1.54	16.4	0.25		1.64	222
1	•	1.98	5.3	0.5	•	1.1	227
1.5	•	0.82	6.6	1		0.96	574
2	•	0.87	8.4	2	•	0.86	421
				3		0.83	272
				6		0.96	339
				9	•	0.88	162
				10	•	1.89	355

Serial creatinine and FK-506 levels of 4 lung transplanted patient. (•) Urine sample was collected at that point of time.

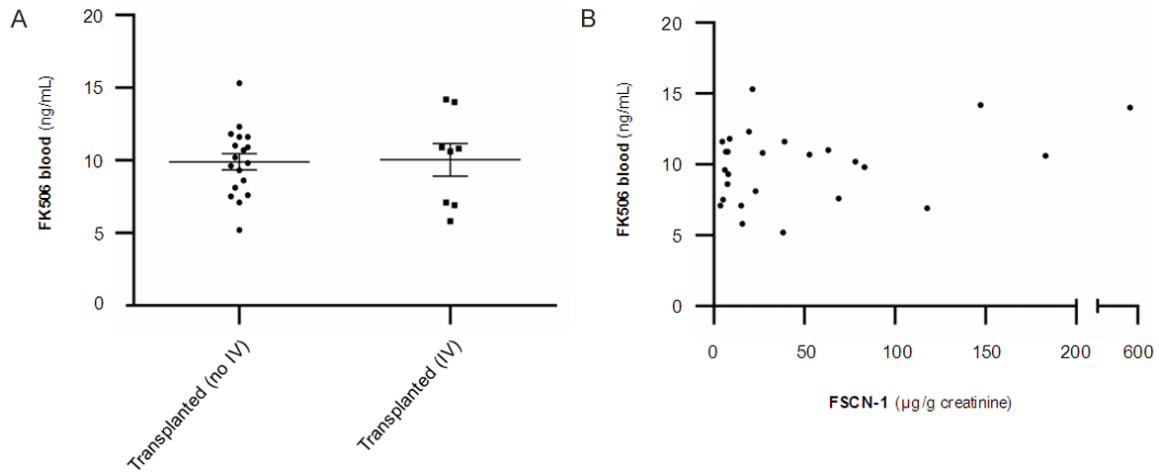


Figure S1. Urinary levels of fascin-1 are not related to systemic blood levels of FK-506. Systemic blood levels of FK-506 were analyzed in kidney transplanted patients attending to isometric vacuolization of tubular cells (A) and urinary fascin-1 levels (B). There were no differences in blood FK-506 levels in kidney transplanted patients showing isometric vacuolization compared with those with normal tubular histology (A) and no relation was observed when correlating urinary fascin-1 levels with blood FK-506 levels ($r^2 = 0.12$, $P > 0.05$) (B). Data were analyzed using a “t” test with Welch correction and correlation was performed with Pearson r coefficient.